10/038,177

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=> s synthesiz###(10a)cDNA (10a)(single strand bind### protein# or E.coli SSB)
L1            0 SYNTHESIZ###(10A) CDNA (10A)(SINGLE STRAND BIND### PROTEIN# OR
                E.COLI SSB)

=>

=> s reverse trancri##### (10a)(SINGLE STRAND BIND### PROTEIN# OR E.COLI SSB)
L2            0 REVERSE TRANCRI##### (10A)(SINGLE STRAND BIND### PROTEIN# OR
                E.COLI SSB)

=> s synthesiz### cDNA and (SINGLE STRAND BIND### PROTEIN# OR E.COLI SSB)
L3            0 SYNTHESIZ### CDNA AND (SINGLE STRAND BIND### PROTEIN# OR E.COLI
                SSB)

=>

=> s reverse transcrip#### and (single strand bind### or E.Coli SSB)
L4           12 REVERSE TRANSCRIP#### AND (SINGLE STRAND BIND### OR E.COLI SSB)

=> s l4 and (synthesiz### (10a) cDNA)
L5            0 L4 AND (SYNTHESIZ### (10A) CDNA)

=> s l4 and synthesiz### and cDNA
L6            0 L4 AND SYNTHESIZ### AND CDNA

=> dup rem l4
PROCESSING COMPLETED FOR L4
L7            6 DUP REM L4 (6 DUPLICATES REMOVED)

=> d l7 1-6 bib ab kwic

L7   ANSWER 1 OF 6     MEDLINE
AN   2001636376     MEDLINE
DN   21546329   PubMed ID: 11690556
TI   Loss of mitochondrial DNA in rabbit bladder smooth muscle following
     partial outlet obstruction results from lack of organellar DNA
     replication.
AU   Wang Z; Wu X; Levin R M; Hudson A P
CS   Department of Immunology and Microbiology, Wayne State University School
     of Medicine, Detroit, Michigan 48201, USA.
NC   DK-47949 (NIDDK)
SO   MOLECULAR UROLOGY, (2001 Autumn) 5 (3) 99-104.
     Journal code: 9709255. ISSN: 1091-5362.
CY   United States
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
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EM 200202
ED Entered STN: 20011107
Last Updated on STN: 20020215
Entered Medline: 20020214
AB When the rabbit bladder outlet is partially obstructed, the relative amount of mitochondrial (mt) DNA per cell in bladder smooth muscle falls rapidly. In order to assess whether this loss of organellar genome results from attenuation of mt DNA replication, we cloned portions of rabbit genes specifying the **single-strand binding** (SSB) protein required for initiation of mt DNA replication, and the catalytic subunit of DNA polymerase gamma (pol gamma), the replication enzyme itself. We then designed primer-probe systems for real-time RT-PCR (TaqMan) analyses for each gene. These were used to assess mRNA in preparations from bladder smooth muscle and mucosa from rabbits subjected to surgical obstruction of the bladder outlet for up to 14 days. mRNA from the pol gamma gene remained essentially at control level in smooth muscle and mucosa in all samples. In mucosa, mRNA from the SSB protein gene remained virtually at control levels in all samples, as did mt genome copy number. In smooth muscle, however, levels of this mRNA declined by >95% within 3 days of obstruction and remained at that level through 14 days; this attenuation of SSB protein mRNA paralleled the loss of mt DNA in the same smooth muscle samples. Thus, lack of mt SSB protein, and consequently attenuated mt DNA replication, is a primary factor in the loss of mt genome copies in bladder smooth muscle after outlet obstruction in the rabbit model of benign bladder dysfunction.
AB . . . this loss of organellar genome results from attenuation of mt DNA replication, we cloned portions of rabbit genes specifying the **single-strand binding** (SSB) protein required for initiation of mt DNA replication, and the catalytic subunit of DNA polymerase gamma (pol gamma), the. . .
CT . . .
DNA Polymerase: GE, genetics
DNA-Directed DNA Polymerase: ME, metabolism
*Muscle, Smooth: PH, physiology
Muscle, Smooth: UL, ultrastructure
Protein Subunits
Rabbits
Reverse Transcriptase Polymerase Chain Reaction

L7 ANSWER 2 OF 6 MEDLINE DUPLICATE 1
AN 1998284107 MEDLINE
DN 98284107 PubMed ID: 9618201
TI Quantitative **reverse transcription** strand displacement amplification: quantitation of nucleic acids using an isothermal amplification technique.
AU Nycz C M; Dean C H; Haaland P D; Spargo C A; Walker G T
CS Department of Molecular Biology, Becton Dickinson Research Center, Research Triangle Park, North Carolina 27709, USA.. nycz@bdrc.bd.com
SO ANALYTICAL BIOCHEMISTRY, (1998 Jun 1) 259 (2) 226-34.
Journal code: 0370535. ISSN: 0003-2697.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; AIDS
EM 199807
ED Entered STN: 19980716
Last Updated on STN: 19980716
Entered Medline: 19980709
AB Recent advances in nucleic acid amplification techniques have allowed for quantitation of viral nucleic acid levels in clinical specimens. The most prevalent testing is carried out for HIV viral load. Strand displacement amplification (SDA) is an isothermal DNA amplification system utilizing a restriction enzyme and a DNA polymerase with strand displacement properties. SDA was adapted for quantitative RNA amplification (QRT-SDA)

of an HIV gag sequence by including AMV **reverse transcriptase**, a quantitative control sequence, and 32P-labeled detector oligonucleotides for the HIV and the control sequences. We have also improved the amplification efficiency by including the **single-strand binding** protein from gene 32 of T4 bacteriophage (T4gp32) to enhance strand displacement replication. In a preliminary analytical demonstration of the technique, RT-SDA was quantitative to within twofold over a range of 500-500,000 transcripts that were generated from a plasmid bearing an HIV gag sequence. QRT-SDA potentially represents a convenient alternative for viral load testing in a clinical setting.
Copyright 1998 Academic Press.

TI Quantitative **reverse transcription** strand displacement amplification: quantitation of nucleic acids using an isothermal amplification technique.

AB . . . with strand displacement properties. SDA was adapted for quantitative RNA amplification (QRT-SDA) of an HIV gag sequence by including AMV **reverse transcriptase**, a quantitative control sequence, and 32P-labeled detector oligonucleotides for the HIV and the control sequences. We have also improved the amplification efficiency by including the **single-strand binding** protein from gene 32 of T4 bacteriophage (T4gp32) to enhance strand displacement replication. In a preliminary analytical demonstration of the. . .

L7 ANSWER 3 OF 6 MEDLINE DUPLICATE 2
AN 97347475 MEDLINE
DN 97347475 PubMed ID: 9201981
TI Evidence for a unique mechanism of strand transfer from the transactivation response region of HIV-1.
AU Kim J K; Palaniappan C; Wu W; Fay P J; Bambara R A
CS Department of Biochemistry and Biophysics, University of Rochester, Rochester, New York 14642, USA.
NC CA 11198 (NCI)
GM 49573 (NIGMS)
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1997 Jul 4) 272 (27) 16769-77.
Journal code: 2985121R. ISSN: 0021-9258.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; AIDS
EM 199707
ED Entered STN: 19970812
Last Updated on STN: 19980206
Entered Medline: 19970731

AB We previously found that strand transfer by human immunodeficiency virus type 1 (HIV-1) **reverse transcriptase** (RT) is promoted at sites where RT pauses during synthesis. In this report, strand transfer is measured within the 5' transactivation response region (TAR) of HIV-1 RNA. We hypothesized that the stable hairpin structure of TAR would induce RT pausing, promoting RNase H-directed cleavage of the template and subsequent transfer at that site. We further predicted that HIV-1 nucleocapsid protein (NC), known to melt secondary structures, would decrease transfer. We show that TAR created a strong pause site for RT, but NC significantly promoted strand transfer. The effect of NC is specific, since other **single strand binding** proteins failed to stimulate transfer. In another unexpected outcome, preferred positions of internal transfer were not at the pause site but were in the upper stem and loop of TAR. Thus, we propose a new mechanism for transfer within TAR described by an interactive hairpin model, in which association between the donor and the acceptor templates within the TAR stem promotes transfer. The model is consistent with the observed stimulation of strand transfer by NC. The model is applicable to internal and replicative end transfer.

AB We previously found that strand transfer by human immunodeficiency virus type 1 (HIV-1) **reverse transcriptase** (RT) is promoted at sites where RT pauses during synthesis. In this report, strand transfer is measured within the 5'. . . a strong pause site for RT, but NC significantly promoted strand transfer. The effect of NC is specific, since other **single strand binding** proteins failed to stimulate transfer. In another unexpected outcome, preferred positions of internal transfer were not at the pause site. . .

CT . . . Human; Support, U.S. Gov't, P.H.S.
Base Sequence
Binding Sites
DNA, Viral: ME, metabolism
*HIV Long Terminal Repeat
*HIV-1: GE, genetics
***HIV-1 Reverse Transcriptase: ME, metabolism**
Models, Genetic
Molecular Sequence Data
Nucleic Acid Conformation
Nucleocapsid: ME, metabolism
Ribonuclease H, Calf Thymus: ME,. . .

CN 0 (DNA, Viral); EC 2.7.7.- (HIV-1 **Reverse Transcriptase**); EC 3.1.26.4 (Ribonuclease H, Calf Thymus)

L7 ANSWER 4 OF 6 MEDLINE DUPLICATE 3
AN 96134828 MEDLINE
DN 96134828 PubMed ID: 8555166
TI Effect of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein on HIV-1 **reverse transcriptase** activity in vitro.
AU Ji X; Klarmann G J; Preston B D
CS Department of Biochemistry, University of Utah, Salt Lake City 84112, USA.
NC P30 ES05022 (NIEHS)
R29 CA48174 (NCI)
RO1 AI34834 (NIAID)
+
SO BIOCHEMISTRY, (1996 Jan 9) 35 (1) 132-43.
Journal code: 0370623. ISSN: 0006-2960.
CY United States
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals; AIDS
EM 199602
ED Entered STN: 19960312
Last Updated on STN: 19980206
Entered Medline: 19960227
AB Conversion of human immunodeficiency virus type 1 (HIV-1) genomic RNA to viral DNA is a requisite step in the virus life cycle. This conversion is catalyzed by **reverse transcriptase** (RT) associated with a large nucleoprotein complex composed of several viral proteins including nucleocapsid (NC). To better characterize the biochemical mechanisms of viral DNA synthesis, we overexpressed and purified recombinant HIV-1 NC and studied its effect on the activity and processivity of HIV-1 RT during polymerization of HIV-1 template sequences in vitro. The effect of NC on steady-state RT activity was dependent on the order of addition of reaction components. Addition of NC prior to formation of RT-primer.template-dNTP ternary complexes inhibited primer extension and reduced total product yields by slowing steady-state RT turnover. In contrast, addition of NC to preformed ternary complexes resulted in efficient primer extension and increased RT processivity at specific DNA template sites. NC stimulated polymerization (2-4 times) through eight of 13 sites examined in the cRRE region of HIV-1 env and increased the rate of polymerization through the D3/CTS region of HIV-1 pol 10 times. The data suggest that NC affects RT processivity by facilitating polymerization through regions of template secondary structure. Thus, NC functions as a **single-strand**

binding (SSB)-like accessory replication factor for RT in vitro and may be part of a multicomponent retroviral replication complex.

TI Effect of human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein on HIV-1 **reverse transcriptase** activity in vitro.

AB . . . (HIV-1) genomic RNA to viral DNA is a requisite step in the virus life cycle. This conversion is catalyzed by **reverse transcriptase** (RT) associated with a large nucleoprotein complex composed of several viral proteins including nucleocapsid (NC). To better characterize the biochemical. . . suggest that NC affects RT processivity by facilitating polymerization through regions of template secondary structure. Thus, NC functions as a **single-strand binding** (SSB)-like accessory replication factor for RT in vitro and may be part of a multicomponent retroviral replication complex.

CT . . . Gov't; Support, U.S. Gov't, P.H.S.
Base Sequence
Binding Sites
*Capsid: PD, pharmacology
DNA Primers
Electrophoresis, Polyacrylamide Gel
*HIV-1: ME, metabolism
HIV-1 Reverse Transcriptase
Macromolecular Systems
Molecular Sequence Data
Molecular Weight
Polymerase Chain Reaction
RNA-Directed DNA Polymerase: CH, chemistry
RNA-Directed DNA. . .

CN 0 (Capsid); 0 (DNA Primers); 0 (Macromolecular Systems); 0 (Recombinant Proteins); 0 (Viral Core Proteins); EC 2.7.7.- (HIV-1 **Reverse Transcriptase**); EC 2.7.7.49 (RNA-Directed DNA Polymerase)

L7 ANSWER 5 OF 6 BIOSIS COPYRIGHT 2003 BIOLOGICAL ABSTRACTS INC.
AN 1994:323305 BIOSIS
DN PREV199497336305
TI Retroviral nucleocapsid proteins possess potent nucleic acid strand renaturation activity.
AU Dib-Hajj, Fadia; Khan, Raza; Giedroc, David P.
CS Dep. Biochem. Biophysics, Texas A and M Univ., College Station, TX 77843-2128 USA
SO Protein Science, (1993) Vol. 2, No. 2, pp. 231-243.
ISSN: 0961-8368.
DT Article
LA English
AB The nucleocapsid protein (NC) is the major genomic RNA binding protein that plays integral roles in the structure and replication of all animal retroviruses. In this report, select biochemical properties of recombinant Mason-Pfizer monkey virus (MPMV) and HIV-1 NCs are compared. Evidence is presented that two types of saturated Zn, NC-polynucleotide complexes can be formed under conditions of low (NaCl) that differ in apparent site-size (n = 8 vs. n = 14). The formation of one or the other complex appears dependent on the molar ratio of NC to RNA nucleotide with the putative low site-size mode apparently predominating under conditions of protein excess. Both MPMV and HIV-1 NCs kinetically facilitate the renaturation of two complementary DNA strands, suggesting that this is a general property of retroviral NCs. NC proteins increase the second-order rate constant for renaturation of a 149-bp DNA fragment by more than four orders of magnitude over that obtained in the absence of protein at 37 degree C. The protein-assisted rate is 100-200-fold faster than that obtained at 68 degree C, 1 M NaCl, solution conditions considered to be optimal for strand renaturation. Provided that sufficient NC is present to coat all strands, the presence of 400-1,000-fold excess nonhomologous DNA does not greatly affect the reaction rate. The HIV-1 NC-mediated renaturation reaction functions stoichiometrically, requiring a saturated strand of DNA

nucleotide:NC ratio of about 7-8, rather than 14. Under conditions of less protein, the rate acceleration is not realized. The finding of significant nucleic acid strand renaturation activity may have important implications for various events of **reverse transcription** particularly in initiation and cDNA strand transfer.

AB. . . is not realized. The finding of significant nucleic acid strand renaturation activity may have important implications for various events of **reverse transcription** particularly in initiation and cDNA strand transfer.

IT Miscellaneous Descriptors
RETROVIRAL REPLICATION; **REVERSE TRANSCRIPTION**; **SINGLE-STRAND BINDING** PROTEIN; ZINC(II) NUCLEOCAPSID-POLYNUCLEOTIDE COMPLEXES

L7 ANSWER 6 OF 6 MEDLINE
AN 87174833 MEDLINE
DN 87174833 PubMed ID: 2436148
TI DNA synthesis arrest sites at the right terminus of rat long interspersed repeated (LINE or L1Rn) DNA family members.
AU d'Ambrosio E; Furano A V
SO NUCLEIC ACIDS RESEARCH, (1987 Apr 10) 15 (7) 3155-75.
Journal code: 0411011. ISSN: 0305-1048.
CY ENGLAND: United Kingdom
DT Journal; Article; (JOURNAL ARTICLE)
LA English
FS Priority Journals
OS GENBANK-X05262
EM 198705
ED Entered STN: 19900303
Last Updated on STN: 19980206
Entered Medline: 19870521

AB An approximately equal to 150-bp GC-rich (approximately equal to 60%) region is at the right end of rat long interspersed repeated DNA (LINE or L1Rn) family members. We report here that one of the DNA strands from this region contains several non-palindromic sites that strongly arrest DNA synthesis in vitro by the prokaryotic Klenow and T4 DNA polymerases, the eukaryotic alpha polymerase, and AMV **reverse transcriptase**. The strongest arrest sites are G-rich (approximately equal to 70%) homopurine stretches of 18 or more residues. Shorter homopurine stretches (12 residues or fewer) did not arrest DNA synthesis even if the stretch contains 11/12 G residues. Arrest of the prokaryotic polymerases was not affected by their respective **single strand binding** proteins or polymerase accessory proteins. The region of duplex DNA which contains DNA synthesis arrest sites reacts with bromoacetaldehyde when present in negatively supercoiled molecules. By contrast, homopurine stretches that do not arrest DNA synthesis do not react with bromoacetaldehyde. The presence of bromoacetaldehyde-reactive bases in a G-rich homopurine-containing duplex under torsional stress is thought to be caused by base stacking in the homopurine strand. Therefore, we suggest that base-stacked regions of the template arrest DNA synthesis.

AB . . . strongly arrest DNA synthesis in vitro by the prokaryotic Klenow and T4 DNA polymerases, the eukaryotic alpha polymerase, and AMV **reverse transcriptase**. The strongest arrest sites are G-rich (approximately equal to 70%) homopurine stretches of 18 or more residues. Shorter homopurine stretches. . . synthesis even if the stretch contains 11/12 G residues. Arrest of the prokaryotic polymerases was not affected by their respective **single strand binding** proteins or polymerase accessory proteins. The region of duplex DNA which contains DNA synthesis arrest sites reacts with bromoacetaldehyde when. . .

=>

10/038,177

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Search Results -

Term	Documents
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CDNAS.DWPI,EPAB,JPAB,USPT.	12182
SINGLE.DWPI,EPAB,JPAB,USPT.	1986205
SINGLES.DWPI,EPAB,JPAB,USPT.	1798
STRAND.DWPI,EPAB,JPAB,USPT.	95252
STRANDS.DWPI,EPAB,JPAB,USPT.	78719
"E.COLI".DWPI,EPAB,JPAB,USPT.	5874
E.COLIS	0
SSB.DWPI,EPAB,JPAB,USPT.	2605
SSBS.DWPI,EPAB,JPAB,USPT.	30
SYNTHESIZ$3	0
(SYNTHESIZ$3 NEAR5 CDNA NEAR5 (SINGLE STRAND BIND$3 OR E.COLI SSB)).USPT,JPAB,EPAB,DWPI.	0

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DB=USPT,JPAB,EPAB,DWPI; PLUR=YES; OP=ADJ			
L14	synthesiz$3 near5 cDNA near5 (single strand bind$3 or E.coli ssb)	0	L14
L13	synthesiz$3 near5 cDNA	6658	L13
L12	reverse transcri$5 near5 (single strand bind$3 or E.coli ssb protein$1)	0	L12
L11	L10 and mRNA	25	L11
L10	l8 and concentration$1	31	L10
L9	L8 and (single strand bind$3 near5 concentration)	0	L9
L8	L7 and escherichia coli	32	L8
L7	L6 and (reverse near5 transcri$5)	46	L7
L6	l5 and gp	84	L6
L5	single strand binding protein$1	96	L5
L4	l3 and single strand binding	0	L4
L3	baugh.in.	596	L3
L2	L1 and single strand binding protein$1	0	L2
L1	hunter.in.	4708	L1

END OF SEARCH HISTORY

End of Result Set

Generate Collection

L11: Entry 25 of 25 File: USPT Dec 15, 1998

DOCUMENT-IDENTIFIER: US 5849547 A
TITLE: Method for nucleic acid amplification by transcription using displacement, and reagents and kit therefor

Brief Summary Text (6):
The usefulness of the search for specific nucleotide sequences is immense, especially for the detection of pathogenic organisms, the determination of the presence of alleles, the detection of the presence of lesions in a host genome and the detection of the presence of a specific mRNA or of the modification of a cellular host. Genetic diseases such as Huntington's disease, Duchenne's myopathy, phenylketonuria and .beta.-thalassemia can be diagnosed by analysing DNA from the individual. Furthermore, the diagnosis or the identification of viruses, viroids, bacteria, fungi, protozoa or some other form of plant or animal life can be carried out by hybridization experiments with nucleic probes.

Brief Summary Text (15):
Other target amplification methods exist which are based on the amplification of transcripts (termed TAS). TAS, which is described in Patent Application No. WO 88/10315, consists of the repetition of a cycle with three stages. The first stage makes it possible to synthesize a cDNA from RNA in the presence of reverse transcriptase and a "hybrid" deoxynucleotide primer containing a specific sequence of phage RNA polymerase promoter. Following the thermal denaturation of the RNA/cDNA heteroduplex, the single-stranded cDNA is replicated by reverse transcriptase in the presence of an anti-sense oligonucleotide primer. The DNA homoduplex thus obtained during this second stage contains a double-stranded promoter to which a phage DNA-dependent RNA polymerase can bind. The third stage then consists of transcribing RNA molecules (from 30 to 1000 per template) which will again be able to serve as template for the synthesis of cDNA and thereby to continue the amplification cycle (Davis et al., 1990. J. Infect. Dis. 162: 13-20).

Brief Summary Text (16):
There are various methods derived from TAS, including Self-Sustained Sequence Replication (termed 3SR), which is described in Patent Application WO 90/06995 and European Patent No. 0,373,960, Nucleic Acid Sequence-Based Amplification (termed NASBA) which is described in Patent Application WO 91/02818 and European Patent No. 0,329,822 and Single Primer Sequence Replication (termed SPSR) which is described in U.S. Patent No. 5,194,370. These methods have in common the combination of three enzymatic activities: RNA- and DNA-dependent DNA polymerase (reverse transcriptase), ribonuclease H (RNase H; Escherichia coli enzyme and/or enzymatic activity associated with reverse transcriptase), and DNA-dependent RNA polymerase (T7 bacteriophage RNA polymerase). These methods are based on the same principle and are carried out at a fixed temperature (from 37.degree. to 45.degree. C.), according to a continuous process of reverse transcription and transcription reactions in order to replicate an RNA target via cDNA. As in the case of TAS, an RNA polymerase (T7 phage) binding site is introduced into the CDNA by the primer used for the reverse transcription stage. However, the denaturation of the RNA/cDNA heteroduplex is carried out isothermally by specific hydrolysis of the RNA of this heteroduplex by RNase H activity. The free cDNA is then replicated from a second oligonucleotide primer by reverse transcriptase. The DNA/DNA homoduplex is transcribed into RNA by T7 RNA polymerase and this RNA can again serve as template for the next cycle.

Drawing Description Text (12):
FIG. 9 represents the analysis of the products of transcription using displacement, by electrophoretic separation on a polyacrylamide gel, transfer onto a membrane and

hybridization, as described in Example 2. The arrow indicates the expected product of transcription, of 263 bases. Lane 1 corresponds to the size marker of 285 base pairs obtained by PCR with the aid of primers A24 (SEQ ID No: 2) and 1028 (SEQ ID No: 3). The tests were carried out in the absence of reverse transcriptase and T7 RNA polymerase (lane 2) or in the presence of enzymes, with three types of primers X, Y and Z (lane 3), without displacement primer Y (lane 4) or without primer Z (lane 5).

Drawing Description Text (13):
FIG. 10 represents the analysis of the products of transcription using displacement, by electrophoretic separation on a polyacrylamide gel, transfer onto a membrane and hybridization, as described in Example 3. Lane 1. corresponds to the size marker of 285 base pairs obtained by PCR with the aid of primers A24 (SEQ ID No: 2) and 1028 (SEQ ID No: 3). Lane 2 corresponds to the target transcription control of 285 base pairs in the presence of T7 RNA polymerase. Lanes 3 to 6 correspond to the tests carried out in the absence of one of the reagents, namely T7 RNA polymerase (lane 3), reverse transcriptase (lane 4), primer Z (lane 5) or the displacement primer Y (lane 6). Lane 7 corresponds to the test in the presence of all the reagents, as described in Example 3.

Detailed Description Text (40):
For the amplification method described above to function, it is necessary that the third and fourth primers, if they are present, comprise at least the 5' portion of the sense sequence of the RNA polymerase promoter. This is the case for the primer D-represented in FIGS. 4, 5, 6 and 7 and for the primer F represented in FIGS. 4, 5 and 6. These FIGS. 4 and 6 show that it is not necessary that primer A and/or B contain a complete sequence of the promoter for the RNA polymerase. The reaction products derived from A and F on the one hand, and B and D on the other hand should contain a functional sense sequence of the RNA polymerase promoter. "Strand displacement activity" designates the phenomenon by which a biological, chemical or physical agent, for example a DNA polymerase, causes the dissociation of a paired nucleic acid from its complementary strand in a direction from 5' towards '3, in conjunction with, and close to, the template-dependent nucleic acid synthesis. The strand displacement starts at the 5' end of a paired nucleic acid sequence and the enzyme therefore carries out the nucleic acid synthesis immediately in 5' of the displacement site. The neosynthesized nucleic acid and the displaced nucleic acid generally have the same nucleotide sequence which is complementary to the template nucleic acid strand. The strand displacement activity may be situated on the same molecule as that conferring the activity of nucleic acid synthesis, and particularly the DNA synthesis, or it may be a separate and independent activity. DNA polymerases such as E. coli DNA polymerase I, Klenow fragment of DNA polymerase I, T7 or T5 bacteriophage DNA polymerase, HIV virus reverse transcriptase are enzymes which possess both the polymerase activity and the strand displacement activity. Agents such as helicases can be used in conjunction with inducing agents which do not possess strand displacement activity in order to produce the strand displacement effect, that is to say the displacement of a nucleic acid coupled to the synthesis of a nucleic acid of the same sequence. Likewise, proteins such as Rec A or Single Strand Binding Protein from E. coli or from another organism could be used to produce or to promote the strand displacement, in conjunction with other inducing agents. For further details and a discussion of strand displacement, consult KORNBERG, A. and BAKER T. A. (1992, DNA Replication, 2nd Edition, pp 113-225, Freeman, N.Y.).

Detailed Description Text (82):
In addition, the double-stranded product (V), if it contains a complete sequence of the RNA polymerase promoter, will give a single-stranded RNA transcription product capable of hybridizing with B and H, and the elongation by reverse transcription, with displacement, of these primers results in the production of a single-stranded DNA for which it is easy to check that by hybridization with A followed by elongation, it also makes it possible to obtain the double-stranded DNA product (VI). Analogous reactions, starting with the double-stranded DNA (V'), also result in the double-stranded DNA product (VI). It can be seen in FIG. 3 that the use of the seventh and eighth primers G and H results in the double-stranded DNA product (VI) which is indeed a polynucleotide which can be used as starting material in the general method which was described above.

Detailed Description Text (117):

Some primers of the present invention comprise homologous sequences. An adjustment of the relative concentrations of the various primers makes it possible to promote differential hybridization for good functioning of the cycles.

Detailed Description Text (119):
The polymerases used in the method of the invention should preferably lack a strand displacement activity. This activity is a well known property of certain DNA polymerases (Sambrook et al., 1989. Molecular Cloning : A Laboratory Manual, 2nd Edition, pp. 5.33-5.35, Cold Spring Harbor Laboratory, Cold Spring Harbor). The properties of DNA polymerases, and especially of the strand displacement activity of some of them are detailed by Kornberg and Baker (1992, DNA Replication, 2nd Edition, pp. 113-225, Freeman, N.Y.). The strand displacement activity was initially demonstrated for the Klenow fragment of DNA polymerase I of Escherichia coli (Masamune and Richardson, 1971. J. Biol. Chem.246: 2692-2701) which confers on this enzyme the capacity to initiate the replication of a nucleic acid from the 3' OH end of a break in a double-stranded DNA. This strand displacement property is limited in the case where the DNA polymerases possess a 5'-3' exonuclease activity (Lundquist and Olivera, 1982. Cell 31 : 53-60). This strand displacement activity has also been demonstrated in thermostable DNA polymerases such as Tli DNA polymerase (Kong et al., 1993. J. Biol. Chem. 268 : 1965-1975). In this case, it was also shown that the mutated forms of this enzyme, not having 5'-3' exonuclease activity, have a greater strand displacement capacity. Strand displacement is not a property common to all DNA polymerases since some of them, like T4 DNA polymerases, are not capable of carrying out, on their own, strand displacement. This strand displacement activity has also been demonstrated for T7 DNA polymerase (Lechner et al., 1983. J. Biol. Chem. 258 : 11174-11184) and for HIV reverse transcriptase (Huber et al., 1989. J. Biol. Chem. 264 : 4669-4678). DNA polymerases having a strand displacement capacity, and more particularly capable of initiating polymerization (from 5' towards 3') from the 3' OH end of a break in a double-stranded DNA (FIG. 1) are useful for carrying out the amplification reaction of the present invention. Preferably, a DNA polymerase lacking 5'-3' exonuclease activity is used for carrying out the amplification cycle since the efficiency of the strand displacement activity is greater in enzymes lacking this activity. The Klenow fragment of DNA polymerase I of Escherichia coli constitutes an example of polymerase lacking 5'-3' exonuclease activity, likewise polymerases such as T4 DNA polymerase, T7 DNA polymerase or Sequenase (US Biochemical), T5 DNA polymerase or Phi29 DNA polymerase could also be used. However, the present invention also comprises the use of DNA polymerases having this 5'-3' exonuclease activity when the latter does not prevent the implementation of the amplification method. In this case, the yield of the amplification reaction can be enhanced by specific inhibition of the 5'-3' exonuclease activity of DNA polymerases under the reaction conditions used.

Detailed Description Text (120):
The present method of amplification requires a reverse transcription stage, in order to recopy an transcribed RNA into cDNA. This stage can in particular be carried out by the use of a reverse transcriptase of the AMV (Avian Myeloblastosis Virus) or MMLV (Moloney Murine Leukemia Virus) types which are generally commercially available. Any other enzyme possessing an RNA-and/or DNA-dependent DNA polymerase activity may be used in the present invention, provided that it has a strand displacement activity. In the opposite case, the strand displacement activity could be conferred by an indicator agent, a helicase or Rec A type activity. The Rec A properties, especially in the process of single stranded DNA reassociation, of strand capture or of strand assimilation are detailed by Mc ENTEE and WEINSTOCK in The Enzymes, vol. XIV, pp. 445-470. The reverse transcription stage can for example be carried out with the aid of Escherichia coli DNA polymerase I since it has been demonstrated that this enzyme also has an RNA-dependent DNA polymerase activity (Ricchetti and Buc, 1993. EMBO 12 : 387-396). The present invention can also use, for this purpose, thermostable RNA-and/or DNA-dependent DNA polymerases such as Taq polymerase or Tth polymerase (for a review on the properties of thermostable DNA polymerases, see Rolf et al., 1992. PCR : Clinical Diagnostics and Research, pp. 224-258, Springer-Verlag, Heidelberg).

Detailed Description Text (121):
As a result of the use of the strand displacement properties of DNA polymerases, or of another associated inducing agent, the present invention does not require nuclease activity, whether endonuclease, exonuclease or ribonuclease activity. In particular, the present invention does not require the use of RNase H activity, common to various

other amplification techniques mentioned above, an activity conferred by certain reverse transcriptases and which has to be supplemented by the addition of Escherichia coli RNase H. In the method of the present invention, it is possible to use MMLV reverse transcriptase, which has a lower RNase H activity than that of AMV (Sambrook et al., 1989. Molecular Cloning : A Laboratory Manual, 2nd Edition, pp. 5.52-5.55, 8.11, 8.17, Cold Spring Harbor Laboratory, Cold Spring Harbor), as RNA- and DNA-dependent DNA polymerase. A form of MMLV reverse transcriptase lacking RNase H activity can also be used in the present invention. The RNase H activity of MMLV reverse transcriptase can indeed be suppressed by deleting a portion of the structural gene for this enzyme (Kotewicz et al., 1988. Nucl. Acids Res. 16 : 265-277), resulting in an enhanced polymerase efficiency compared with the wild-type MMLV reverse transcriptase, and commercially available under the name of "Superscript" (Gerard et al., 1989. Focus 11 : 66-69). The RNase H activity of reverse transcriptase can also be suppressed by performing points mutations in the portion of the gene conferring this polymerase activity (Gerard et al., 1992. Focus 14 : 91-93), resulting in an enzyme also having an enhanced efficiency and a DNA polymerization level greater than Superscript. This enzyme is also commercially available under the name of "Superscript II" (GIBCO-BRL). Another form exists which is commercially available under the name of "StrataScript" (STRATAGENE).

Detailed Description Text (139):
The nature and the length of the primers used in this invention, the sequences of promoters used and the RNA polymerase concentrations relative to each type of promoter can be chosen so as to favour one amplification route relative to another, so as to obtain preferentially one form or other of DNA or RNA. It is thus possible to predominantly obtain a single-stranded RNA directly detectable in the methods of detecting the amplification products, downstream of the present invention, without a nucleic acid denaturation stage.

Detailed Description Text (140):
The RNA derived from one of the two routes of the amplification method described (FIG. 5) being a substrate for the second, and vice versa, it therefore seems that the method according to the invention is a cyclic amplification technique in which the accumulation of the reaction products occurs exponentially. Each transcription stage using a promoter makes it possible to obtain, using a DNA template, between 500 and 1000 RNA copies. Each RNA makes it possible to obtain a cDNA copy which will result in a DNA template which is available for the transcription of 500 to 1000 RNA copies complementary to the former. The result is therefore that in a single cycle of the said amplification method, there will be a multiplication of the target sequence by a factor of 2.5.times.10.sup.5 to 10.sup.6. For a minimum reaction time for the amplification method (for example one hour or more), it will be possible to produce several reaction cycles, until the amplification reagents such as nucleoside triphosphates and the primers are exhausted, resulting in an amplification whose yield corresponds to 10.sup.9 to 10.sup.12 DNA and RNA molecules produced for a single initial target molecule. Depending on the concentrations of the reagents used, and especially the various primers, it is possible to favour, by the amplification method, the production of either form of nucleic acids (DNA and/or RNA), and either of the strands of the starting target nucleic acid.

Detailed Description Text (146):
The feasibility of the amplification technique is demonstrated by separating the various stages involved in it. Since the principle of the method is based on a transcription reaction using displacement, the study of the transcription of a DNA target after displacement has been carried out (FIG. 2). The study model chosen is the sequence of the tem gene encoding .beta.-lactamase, an enzyme which confers resistance to the antibiotic ampicillin. The sequence of this gene is described as sequence ID No. 1. This sequence is present in the cloning vector pBR322. This nucleic acid can be obtained by extraction of plasmids from a bacterial culture followed by digestion with restriction endonucleases, or it can be prepared by an appropriate amplification technique (Sambrook et al. 1989. Molecular Cloning : A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor). The feasibility of installing an RNA polymerase promoter by strand displacement, for the production of RNAs complementary to a target sequence (RNA or DNA) by transcription in homogeneous phase (FIG. 2), has been studied for various DNA polymerases. In this specific case, the RNA polymerase used is that of the T7 phage. The tests are carried out in a final volume

of 50 .mu.l and in the 1.times. reaction buffer described by Milligan et al. (1987. Nucl. Acids Res. 15 : 8783-8798) for the use of T7 RNA polymerase, in the presence of DATP, dCTP, dGTP and dTTP (1 mM each, Pharmacia), of ATP, CTP, GTP and UTP (4 mM each, Boehringer), of 1 U/.mu.l of RNAguard (Pharmacia). The quantity of initial target used is 10.sup.11 copies per test, the concentration of T7 RNA polymerase (New England Biolabs) is 1 U/.mu.l and that of DNA polymerase is 0.1 U/.mu.l. The promoter primer A24 (SEQ ID No: 2) containing a consensus sequence of the T7 phage promoter juxtaposed with a sequence complementary to the target, as well as primer Z, called 1028 (SEQ ID No: 3), are, when present, at a final concentration of 500 nM. The displacement primer Y, called DIS1 (SEQ ID No: 4), is at a variable concentration ranging from 0 to 5 .mu.M. The various reagents, except the enzymatic mixture which is likely to be inactivated by heat (DNA polymerase, RNA polymerase and RNAguard) are brought into contact with the target and denatured for 3 minutes at 95.degree. C. (initial target consisting of double-stranded DNA) or 65.degree. C. (initial target consisting of single-stranded RNA), then cooled on ice for the addition of the enzymatic mixture. The glycerol concentration, due to the addition of the enzymes, is equal to 5%. A 2 hour incubation at 37.degree. C. is then carried out, before stopping the reaction by freezing at -20.degree. C.

Detailed Description Text (149):
The membranes are prehybridized by incubating at 37.degree. C. for 60 minutes in 4 ml of 0.1M sodium phosphate buffer pH 7.0 containing 0.5M sodium chloride, 1 mM EDTA, 0.65% SDS, 0.14 mg/ml salmon DNA and 2% polyethylene glycol 6000 (PEG). The hybridization is carried out by incubating for 60 minutes at 37.degree. C. in 5 ml of the same buffer containing, at a concentration of 200 ng/ml, the oligonucleotide A28 (SEQ ID No: 5), complementary to the expected product of transcription (corresponding to the strand of SEQ ID No: 1) of the tem gene, and labelled with horseradish peroxidase by coupling with 5' according to the method described earlier in International Patent WO 91/19812. After 3 washes of 30 seconds in 50 ml of 1X PBS buffer (Sambrook et al., 1989. Molecular Cloning : A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor) containing 0.05% Tween 20, the hybridized oligonucleotide is revealed by the peroxidase activity in the presence of 10 mg of diaminobenzidine tetrahydrochloride dihydrate (DAB) in 20 ml of 20 mM sodium phosphate buffer pH 7.2, containing 150 mM sodium chloride, 2 mg/ml bovine serum albumin. After incubation at room temperature and protected from light for 15 minutes, the reaction is stopped by rinsing with distilled water.

Detailed Description Text (150):
Tests of transcription dependent on displacement were carried out using a target consisting of the plasmid pBR322 previously cleaved with the endonuclease AluI and then purified (10.sup.11 copies/test). The DNA polymerase used is a DNA-dependent DNA polymerase, the Klenow fragment (Boehringer, 5 U/test), in the absence or in the presence of various concentrations of displacement primer Y, called DIS1 (SEQ ID No: 4), namely: 5 .mu.M (about 10.sup.14 copies), 500 nM, 50 nM, 5 nM or 0.5 nM. A control for T7 RNA polymerase activity was previously made by preparing a double-stranded DNA fragment by PCR, from the AluI-cleaved pBR322 target, and with the aid of primers A24 (SEQ ID No: 2) and 1028 (SEQ ID No: 3). Of course, the double-stranded fragments with defined ends comprising at least one promoter, can be prepared for example by cloning a sequence into a plasmid containing a promoter, digesting with restriction enzymes and purifying on agarose gel followed by electroelution. The 285 base pair product thus obtained contains, in this case, the T7 phage promoter at one end and its transcription by T7 RNA polymerase makes it possible to obtain an RNA of 263 bases corresponding to the reaction product expected during the tests of transcription dependent on displacement. The marker for T7 RNA polymerase activity is made by incubating 10.sup.11 copies of the 285 base pair fragment under the conditions described above, but in the absence of DNA polymerase and dNTPs. This reaction is carried out for two hours, in parallel with the displacement tests.

Detailed Description Text (152):
The results also show that the intensity of the final transcription product varies as a function of the concentration of the displacement primer Y in the tests. The quantity of transcribed material being proportional to the quantity of template available for the T7 RNA polymerase, it therefore appears that the quantity of template synthesized by displacement and extension differs as a function of the quantity of displacement primer Y used in a test. The concentration of primer Z being

constant and in excess, the quantity of double-stranded template available depends on that of the single-stranded product of extension of the promoter primer. It is therefore the case that the quantity of extension product released (single strand) varies as a function of the concentration of displacement primer in the tests. Under the conditions used, a displacement primer concentration of 50 nM (that is to say 10 times the quantity of target present), corresponding to a displacement primer/promoter primer ratio equal to 1/10, gives the best yield of displacement and therefore of transcription. These results show the influence of the displacement primer/promoter primer ratio for the yield of displacement of a nucleic acid strand by extension of a primer situated upstream of the latter. On either side of this optimum, the displacement yield decreases substantially: if the concentration of displacement primer increases, the thermodynamic conditions favour the hybridization of the latter with the target and therefore its extension, at the expense of the hybridization, and then extension of the promoter primer; likewise, the results show that if the displacement primer concentration decreases, the probability of hybridization of the latter upstream of the promoter primer decreases and consequently the probability of extension for the displacement of the strand situated downstream decreases.

Detailed Description Text (155):
The feasibility of the present invention also implies that the transcription in the homogeneous phase, as described in Example 1, can also be carried out using an RNA molecule. This RNA molecule may be either the initial target, the determination of whose presence by amplification is made possible by the present invention, or an intermediate product of the cycle of the amplification method (FIG. 5). This intermediate product may be, by way of example, the RNA obtained by transcription in the case of Example 1. This feasibility presupposes the use of a DNA polymerase, both RNA- and DNA-dependent, which has a strand displacement capacity. In order to demonstrate this feasibility, RNAs, that is to say corresponding to the sequence complementary to the tern sequence (SEQ ID No: 1), were synthesized in vitro with the aid of T7 RNA polymerase, under the reaction conditions of the MEGAscript kit (Ambion), using 10.sup.12 copies of DNA template. In order to do this, a double-stranded DNA template was synthesized by PCR, from pBR322, and with the aid of PROANTI (SEQ ID No: 6) and ORF1 (SEQ ID No: 7) primers. The 889 base pair product thus obtained contains, in this case, at one end, the T7 phage promoter and its transcription by T7 RNA polymerase makes it possible to obtain an RNA of 867 bases corresponding to the sequence complementary to the sequence of the tern gene (SEQ ID No: 1). The synthesized RNAs are treated with DNase I in order to remove the template DNA, purified by phenol/chloroform extraction and precipitated with ethanol in the presence of ammonium acetate salts. The RNAs thus obtained are taken up in water previously treated with diethyl pyrocarbonate (DEPC), analysed by denaturing polyacrylamide gel electrophoresis and assayed by absorbance at 260 nm. A quantity equivalent to 10.sup.11 copies of target RNA (5 nM final) is used per test and the reaction conditions are identical to those described in Example 1, in the presence of the primers X, Y and Z described above. However, the DNA polymerase used within the framework of these tests is the AMV virus reverse transcriptase (Seikagaku) and the displacement primer/promoter primer ratio set at the optimum determined above (1/10) was used, which corresponds to a final displacement primer concentration equal to 50 nM final. The analysis of the reaction products is carried out as described in Example 1, by electrophoretic separation, transfer onto a membrane and hybridization with the aid of probe A28 (SEQ ID No: 5) labelled with horseradish peroxidase. FIG. 9 illustrates the results obtained during the different reaction tests. A quantity equivalent to 10.sup.12 copies of PCR fragment of 285 base pairs, containing a T7 promoter at one end, obtained with the aid of primers A24 (SEQ ID No: 2) and 1028 (SEQ ID No: 3), was loaded (lane 1) to serve as size marker for the expected RNA (263 bases), corresponding to the transcription of an identical fragment, during the tests carried out. The tests were carried out in the absence of reverse transcriptase and T7 RNA polymerase (lane 2), and in the presence of enzymes, with primers X, Y and Z (lane 3), without displacement primer Y (lane 4) or without primer Z (lane 5). The expected RNA of 263 bases is detected only from the test containing all the enzymatic reagents and the primers, whereas in the absence of displacement primer, no transcription product is obtained. This result therefore confirms the feasibility of the strand displacement by the action of a DNA polymerase and demonstrates the feasibility of the displacement on an RNA template, by virtue of the strand displacement capacity of AMV reverse transcriptase. The latter is therefore capable, during the extension of a primer situated upstream of a previously extended primer, of separating an RNA-DNA

heteroduplex. The stability of the latter being known to be greater than that of a DNA-DNA homoduplex, it is a fortiori likely that this reverse transcriptase is capable, by primer extension, of displacing a DNA strand hybridized with a DNA target. Although an RNase H activity associated with-AMV reverse transcriptase is described, no transcription product is obtained in the absence of displacement primer. This suggests that the buffer conditions used in these tests are not appropriate for this RNase H activity or that the sequence of the RNA target used is not very sensitive to the action of the latter. The RNA of the target RNA-CDNA duplex cannot therefore be degraded and the product of extension of the promoter primer cannot therefore be released for the hybridization and the extension of primer Z on the latter. This result therefore demonstrates, in addition to the strand displacement capacity of AMV reverse transcriptase, the total lack of efficiency, under these conditions, of the separation of a cDNA from a cDNA-RNA heteroduplex by the action of the RNase H associated with the AMV reverse transcriptase, justifying the use of exogenous Escherichia coli RNase H in the transcriptional amplification methods described above, such as NASBA, 3SR or LAT. In the absence of primer Z (but in the presence of displacement primer Y), no transcription product is obtained, which shows that no self-priming reaction occurs at the 3' end of the cDNA derived from the extension of the promoter primer, under the conditions of the present reaction, although this property has been described in reverse transcriptases under specific conditions.

Detailed Description Text (156):
This example therefore demonstrates the efficiency of the method for installing an RNA polymerase promoter by strand displacement for the production of RNAs complementary to an RNA target sequence, by transcription in a homogenous phase (a single stage and a single temperature) as described in FIG. 2, and to produce multiple RNA strands complementary to a given target RNA strand. The method is therefore composed of a single stage, without subsequent or intermediate addition of reagents, and requires no nuclease, in particular RNase H, activity for the separation of a cDNA from an RNA-DNA heteroduplex. In particular, under the reaction conditions, AMV reverse transcriptase shows no RNase H activity which would allow the release, in the absence of displacement primer, of the strand extended from the promoter primer on an RNA template. The strand displacement capacity of AMV reverse transcriptase therefore demonstrates the feasibility of the amplification cycle and consequently the exponential nature of the accumulation of the reaction products from an RNA or a DNA target (FIG. 5).

Detailed Description Text (158):
In order to confirm the feasibility of the separation of a DNA molecule from a DNA-RNA heteroduplex by strand displacement, and this independently of any residual RNase H activity intrinsic to certain reverse transcriptases, tests were carried out using an MMLV reverse transcriptase lacking RNase H activity. Such an enzyme, obtained by genetic engineering, is commercially available under the name "Superscript.sup.II " (GIBCO-BRL). The tests were carried out as in Example 2, using the same RNA target, with the aid of the primers X, Y and Z described above. Superscript.sup.II is added to a final concentration of 4 U/.mu.l (200 U/test) and the displacement and promoter primers are present at a ratio of 1/10. The reaction products are analysed in the manner described in Example 1. The results obtained are presented in FIG. 10. It appears that the presence of a transcription product is limited to the sample containing a displacement primer Y (lane 7), which confirms the strand displacement capacity of MMLV reverse transcriptase lacking RNase H activity. In the absence of displacement primer (lane 6), no transcription signal is obtained, confirming the validity of the separation of a DNA from an RNA-DNA heteroduplex by the strand displacement properties of reverse transcriptases. In the absence of primer Z (lane 5), no transcription product is obtained, which confirms, as in Example 2, that the MMLV reverse transcriptase "Superscript.sup.II " does not carry out the self-repriming of the 3' end of the CDNA under these reaction conditions.

Detailed Description Text (159):
This example therefore confirms the feasibility of the method of amplification by transcription using displacement, from an RNA target, and this in a manner completely independent of the RNase H activities which may be present in the reaction medium. The amplification method described in the present invention can therefore be carried out with the aid of a reverse transcriptase and an RNA polymerase, without any other additional enzymatic activity.

Detailed Description Text (161):
The feasibility of the amplification method described in the present invention also depends on the capacity of RNA polymerases to transcribe complementary RNAs from one and the same double-stranded template containing functional RNA polymerase promoters at each of its ends, in particular in the method described in FIGS. 3 and 5. In order to validate this hypothesis, a double-stranded DNA template was synthesized by PCR, in the presence of primers DTA1 (SEQ ID No: 8) and DTA2 (SEQ ID No: 9), from the target pBR322. The 275 base pair fragment thus obtained is purified. It contains a portion of the sequence of the ten gene (from nucleotide 312 to 486 inclusive of SEQ ID No: 1), flanked by two promoters for T7 phage RNA polymerase, orientated for the transcription of either of the strands of the ten sequence. Each promoter sense sequence is preceded in 5' by a sequence corresponding to primer F220 (SEQ ID No: 10). The transcription of such a fragment from each of the promoters situated at its ends should theoretically result in the synthesis of two RNA populations complementary to each other and containing at their 3' end the antisense sequence of the T7 promoter (that is to say the sequence complementary to the sense sequence of the T7 promoter) followed by the sequence complementary to the oligonucleotide F220 (SEQ ID No: 10). The size of the expected RNAs is 231 bases, and they contain the sequence between nucleotides 312 and 486 (inclusive) of the tem gene (SEQ ID No: 1), or the sequence complementary to the latter. Transcription tests are carried out, using the PCR fragment containing a promoter at each end, in the 1.times. buffer described by Milligan et al. (1987. Nucl. Acids Res. 15 : 8783-8798), in a final volume of 50 .mu.l, in the presence of ATP, CTP, GTP and UTP (4 mM each), of 1 U/.mu.l of T7 phage RNA polymerase (New England Biolabs) and 1 U/.mu.l of RNAguard (Pharmacia). The PCR template is used at 10.sup.11 copies per test and the glycerol concentration due to addition of the enzymes is 5%. The transcription is carried out for 2 hours at 37.degree. C. and stopped by freezing to -20.degree. C. The reaction product (10 .mu.l) is analysed by denaturing polyacrylamide gel electrophoretic separation and then transferred onto a nylon membrane, as described in Example 1. Two membranes are thus prepared in order to carry out a hybridization of probe A28 (SEQ ID No: 5) labelled in 5' with horseradish peroxidase, on the one hand, and of probe A19 (SEQ ID No: 11) also labelled in 5' with horseradish peroxidase on the other hand. Probe A19 makes it possible to detect the RNAs complementary to the tem sequence described (SEQ ID No: 1), whereas probe A28 allows the detection of the RNAs corresponding to the tem sequence described (SEQ ID No: 1). FIG. 11 illustrates the results obtained during these tests. Probes A28 and A19 make it possible to detect 10.sup.12 copies of double stranded target DNA template of 275 base pairs (lane 1), as well as RNAs of 231 bases in the case of transcriptional tests (lane 2). The detection of the expected transcripts with the aid of the two types of probes A19 (part A) and A28 (part B) show that the transcription of complementary RNAs was carried out by T7 RNA polymerase, from each promoter of the DNA template. The production of complementary RNAs from a common DNA template containing a promoter at each of its ends demonstrates that T7 RNA polymerase is capable of carrying out the polymerization of RNAs by convergent progression on the same template. The possibility of producing complementary RNAs under these conditions with the aid of RNA polymerase therefore confirms the feasibility of the amplification method of the present invention described in FIGS. 3 and 5. These results therefore confirm that the amplification cycle described in the present invention in FIGS. 3 and 5 has an exponential character, resulting in a substantial accumulation of RNA and DNA of which the sequence corresponds to the portion of the target between the promoter primers A and B (FIG. 5).

Detailed Description Text (163):
The experimental validity of the DTR amplification method was proved by studying one of the amplification cycles described in the present invention, and in particular as described in FIG. 15. In order to do this, a PCR fragment of 167 base pairs was synthesized with the aid of primers DTA7 (SEQ ID No: 12) and DTA8 (SEQ ID No: 13), from pBR322, and which therefore contains a portion of the sequence of the tem gene (from nucleotide 336 to 402 inclusive of SEQ ID No: 1). This PCR fragment corresponds to one type of molecule for entry into the amplification cycle and therefore contains at each of its ends an RNA polymerase promoter sequence (in particular the T7 phage promoter), preceded in 5' by a defined sequence such as the F220 sequence (SEQ ID No: 10). In order to demonstrate the exponential character of the method, various amplification reactions were carried out in parallel in the presence of decreasing quantities of target molecule consisting of the preceding purified PCR fragment

(corresponding to the entry into the amplification cycle): from 10.sup.10 to 10.sup.6 copies per test. The reactions were carried out in a final volume of 50 .mu.l, in the presence of ATP, CTP, GTP and UTP (4 mM each), of DATP, dCTP, dGTP and dTTP (1 mM each) of 1 U/.mu.l of T7. phage RNA polymerase (New England Biolabs), of 4 U/.mu.l of MMLV reverse transcriptase "Superscript.sup.II " (GIBCO-BRL), lacking RNase H activity, and 1 U/.mu.l of RNAguard (Pharmacia). The reaction medium contained, in addition, the various promoter primers A and B corresponding to DTA7 and DTA8 (SEQ ID No: 12 and SEQ ID o: 13, respectively) at a concentration of 0.01 .mu.M and the displacement primer C equivalent to E corresponding to F220 (SEQ ID No: 10) at a concentration of 1 .mu.M. After incubating for two hours at 37.degree. C., the amplification reactions are stopped by freezing the reaction medium to -20.degree. C. A fraction of 5 .mu.l, that is to say 1/10 of the reaction volume, is quantitatively analysed by capture and specific detection according to the ELOSA method (Enzyme Linked Oligo Sorbent Assay). The method involves the attachment of a capture oligonucleotide onto a solid support (microtitre plate), the denaturation of the reaction product, its hybridization with the capture probe specific for the amplified sequence and revealing by a detection probe coupled to horseradish peroxidase. The capture oligonucleotide A20 (SEQ ID No: 14) is passively attached onto the wells of a Maxisorp Nunc-Immuno plate microtitre plate according to the method already described in Patent FR 91 09057, allowing the attachment of about 5 pmol of oligonucleotide in one well. After attachment, three washes are carried out with 1.times. PBS-Tween buffer. The detection of the captured amplification product is carried out in accordance with the technique described earlier in Patent FR 91 09057. The 5 .mu.l fraction to be analysed is added to a volume of 35 .mu.l of 0.2M sodium phosphate buffer pH 7.0, containing 1M sodium chloride, 2 mM EDTA, 1.3% SDS, 0.24 mg/ml salmon DNA, 4% polyethylene glycol (PEG) 6000. The nucleic acids contained in this sample are denatured by addition of 5 .mu.l of 2M sodium hydroxide at room temperature, and neutralized after 3 minutes by addition of 5 .mu.l of 2M acetic acid. As control and for calibration, two RNA dilution series corresponding to the expected amplification products are prepared. The samples are then loaded into the well of a microtitre plate in which the oligonucleotide capture probe A20 (SEQ ID No: 14) has been previously attached. The amount loaded is either 50 .mu.l of undiluted sample or the same volume of sample diluted 1/10 or 1/100. Immediately, a volume of 50 .mu.l of 0.2M sodium phosphate buffer pH 7.0, containing 1M sodium chloride, 2 mM EDTA, 1.3% SDS, 0.24 mg/ml of salmon DNA, 4% polyethylene glycol (PEG) 60QO and 5 ng of detection oligonucleotide probe A18 (SEQ ID No: 15) coupled to horseradish peroxidase, is added. After incubating for 60 minutes at 37.degree. C., the wells are washed with 1.times. PBS-Tween. The revealing of the peroxidase-A18 probe hybridized with the amplification product is carried out by addition of 100 .mu.l of a solution containing the substrate ortho-phenylenediamine (OPD). The calorimetric reaction is stopped after 20 minutes by addition of 100 .mu.l of 1M sulphuric acid. The optical density at 492 nm is read using an AXIA microreader (BioMerieux). The results obtained are represented by the histograms in FIG. 18. For each of the target dilutions, three tests were carried out: complete test (as described above), test without displacement primer F220 (SEQ ID No: 10) and test without MMLV reverse transcriptase "Superscript.sup.II ". The results show that the amplification method (complete system) makes it possible to detect under these conditions a significant specific signal down to an initial target quantity of 10.sup.7 copies per test, that is to say a sensitivity of 10.sup.6 copies, since a fraction equivalent to 1/10th of the reaction medium is analysed under these conditions. This sensitivity is only relative and can be greatly increased if a revealing system other than colorimetry is used (for example fluorescence, chemiluminescence or bioluminescence). The results show in particular that in the absence of displacement primer F220, a specific signal is obtained only for a target quantity equal to 10.sup.10 copies per test. The difference in detection sensitivity between "with" and "without" displacement primer is therefore a factor of 10.sup.3, or of 3 Log of base 10 units. This demonstrates that the method allows a substantial accumulation of the amplification product, and this can only be obtained by carrying out the amplification cycle. This cycling is therefore only possible because of the presence of a displacement primer such as primer F220. Likewise, if the MMLV reverse transcriptase "Superscript.sup.II " is suppressed, a detection signal is only obtained for an initial target quantity greater than or equal to 10.sup.10 copies per test. These latter reaction conditions in fact correspond to a transcription stage carried out on the bifunctional molecule corresponding to the entry into the amplification cycle, as described in Example 4. These data therefore show that the method is vastly more sensitive than transcription alone and that this sensitivity depends on the

cycling of the method up to the transcription stage using an enzymatic displacement stage, in particular with the aid of a displacement primer in the presence of a DNA polymerase such as a reverse transcriptase. Qualitative analysis of the amplification tests by electrophoretic separation, transfer onto a nylon membrane and hybridization, as described in Example 1, with the aid of probe A28 (SEQ ID No: 5) on the one hand and of probe A18 (SEQ ID No: 15) on the other hand, coupled to horseradish peroxidase, made it possible to detect two complementary RNA molecules of 120 bases, corresponding to the expected amplification product (117 bases).

Detailed Description Text (167):
The "DTR" amplification method described in the present invention is based on an exponential cycle using, in a combined manner, transcription and strand displacement. In particular, the enzymatic strand displacement calls into play, as described in FIG. 15, a reverse transcriptase, a displacement primer C (equivalent to E in this example) and two promoter primers (A and B) which are to be displaced. Within the amplification cycle, the displacement primer/displaced primer (promoter primer here) ratio greatly influences the amplification yield of the method. If the amplification is carried out under the preceding reaction conditions, in the presence of 10.sup.8 or 10.sup.9 copies of initial target, with a fixed concentration of promoter primers A and B corresponding to DTA7 (SEQ ID No: 12) and DTA8 (SEQ ID No: 13) equal to 0.1 .mu.M, and a concentration of displacement primer C corresponding to F220 (SEQ ID No: 10) equal to 0.001, 0.01, 0.1, 1 or 10 .mu.M, an amplification yield is obtained which increases with the displacement primer/displaced primer ratio (FIG. 19). Quantitative analysis on a microtitre plate, as described in Example 5, using a capture probe A25 (SEQ ID No: 17) and a detection probe A28 (SEQ ID No: 5), shows (FIG. 19) that the displacement primer/displaced primer ratio equal to 100 is the most favourable in this case (it will be noted that a value equal to 2500 corresponds to an optical saturation of the apparatus and therefore corresponds to a signal greater than or equal to 2500). This ratio should be adjusted according to the length of the displacement or promoter primers used, or according to the nature of the target sequence, in order to promote the cycling stage by enzymatic strand displacement.

Detailed Description Text (171):
The "DTR" amplification method of the present invention results essentially in a double-stranded RNA corresponding to a portion of the target sequence. In order to facilitate the detection of this double-stranded RNA by hybridization methods, or in special cases of production of single-stranded RNA, it may be useful to favour the appearance of an RNA strand at the expense of its complementary. This can be carried out by the combined use of two types of promoter primers A and B comprising a different promoter for phage (for example T7 and T3) RNA polymerase, in the presence of unbalanced RNA polymerase concentrations. For example, we synthesized by PCR, a double-stranded DNA molecule of 167 base pairs corresponding to the entry into one of the amplification cycles of the present invention (FIG. 15) using primer DTA8 (SEQ ID No: 13) comprising a T7 promoter and DTA9 (SEQ ID No: 18) containing a T3 promoter. Amplification tests were carried out on dilutions of this target in accordance with Example 5, in the presence of T7 RNA polymerase concentrations ranging from 0 to 50 U/test and a constant T3 RNA polymerase concentration equal to 50 U/test. FIG. 21 shows that the use of the enzyme ratio 6.25 U of T7 RNA polymerase to 50 U of T3 RNA polymerase makes it possible to obtain, by capture and detection with the aid of probes A25 (SEQ ID No: 17) and A28 (SEQ ID No: 5) respectively, an increased amplification signal and results in a significant signal down to a target quantity of 10.sup.5 copies per test, which corresponds to a sensitivity of 10.sup.4 copies of target molecules, by calorimetric revealing. The multiplication factor for the target amplification method, knowing that the sensitivity of the detection method is 10.sup.10 copies, is therefore 10.sup.5 ; thereby conferring on the method an efficiency which makes it useful in numerous applications.

CLAIMS:

1. Method of amplifying a sequence of a target nucleic acid, said sequence comprising, from its 5' end, in the 5'-3' direction, an upstream sequence having at least 5 nucleotides and from its 3' end in the 3'-5' direction, a downstream sequence having at least 5 nucleotides,

said method comprising the steps of:

obtaining a polynucleotide comprising a first segment corresponding to the sequence to be amplified and at least a second segment comprising the sense sequence of a first RNA polymerase promoter or the antisense sequence of a second RNA polymerase promoter or at least a portion of said sense or antisense sequence, wherein such a second segment comprising said sense sequence of the first promoter or portion thereof is situated upstream of the 5' end of said first segment, and such a second segment comprising said antisense sequence of the second promoter or portion thereof is situated downstream of the 3' end of said first segment, and

bringing said polynucleotide into contact with an excess amount of a set of primers, in the presence of a system having RNA polymerase activity, RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and strand displacement activity, under conditions allowing the function of the activities, and in the presence of an excess amount of deoxyribonucleoside triphosphates and ribonucleoside triphosphates, such that said sequence of the target nucleic acid is amplified, said set of primers comprising:

a) at least one primer selected from the group consisting of:

1) a first primer comprising successively from its 5' end towards its 3' end:

a first optional polynucleotide segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of said first RNA polymerase promoter including its 3'-terminal portion,

and a third segment having the same length as said upstream sequence and being either homologous to said upstream sequence, or capable of hybridizing with said upstream sequence, and

2) a second primer comprising successively, from its 5' end towards its 3' end:

a first optional segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of said second RNA polymerase promoter including its 3'-terminal portion,

and a third segment having the same length as said downstream sequence and being either homologous to said downstream sequence, or capable of hybridizing with said downstream sequence,

wherein, when said first and second primers are both present, the third segment of one of the first and second primers is homologous to one of the upstream or downstream sequences of said sequence to be amplified and the third segment of the other primer is capable of hybridizing with the other downstream or upstream sequence, and

b) at least one primer selected from the group consisting of:

1) a third primer comprising at its 3'-end:

either a sequence containing a segment that is homologous to all or part of the second segment of the first primer and containing the 5'-end of said sense sequence of said first RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of the first primer but not comprising its 5' end,

2) a fourth primer comprising at its 3'-end:

either a sequence containing a segment that is homologous to all or part of the second segment of the second primer and containing the 5'-end of said sense sequence of said second RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of the second primer but not comprising its 5'-end,

3) a fifth primer comprising at its 3'-end:

either a sequence homologous to a portion of the third primer, said portion not comprising the 3'-terminal nucleotide of said third primer, or a sequence homologous to at least a portion of said first segment of the first primer,

wherein, when said third and fifth primers are both present, the 3'-end of the third primer hybridizes downstream of the 3'-end of the fifth primer, and

4) a sixth primer comprising at its 3'-end:

either a sequence homologous to a portion of the fourth primer, said portion not comprising the 3'-terminal nucleotide of the fourth primer,

or a sequence homologous to at least a portion of said first segment of the second primer,

wherein, when said fourth and sixth primers are both present, the 3'-end of the fourth primer hybridizes downstream of the 3'-end of the sixth primer.

2. Method according to claim 1, wherein said polynucleotide further comprises at least one third segment selected from the group consisting of:

a) upstream of the 5' end of a said second segment comprising the sense sequence of said first promoter or portion thereof, a segment homologous to the first segment of either the first primer or the second primer, and

b) downstream of the 3' end of a said second segment comprising the antisense sequence of said second promoter or portion thereof, a segment capable of hybridizing with the first segment of either the first primer or the second primer.

4. Set of primers for amplifying a sequence of a target nucleic acid, comprising:

a) at least one primer selected from the group consisting of:

1) a primer A1 comprising successively, from its 5' end towards its 3' end:

a first optional polynucleotide segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of a first RNA polymerase promoter including its 3'-terminal end,

and a third segment capable of hybridizing with a target sequence, and

2) a primer B1 comprising successively, from its 5' end towards its 3' end:

a first optional polynucleotide segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of a second RNA polymerase promoter including its 3'-terminal end,

and a third segment capable of hybridizing with a nucleotide sequence complementary to a sequence of the target situated, on the target, upstream of the sequence with which primer A1 is capable of hybridizing; and

b) at least one primer selected from the group consisting of:

1) a primer A2 comprising at its 3'-end:

either a sequence homologous to all or part of the second segment of the primer A1,

and comprising the 5' end of the sense sequence of said RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of said primer A1 or B1, but not comprising its 5' portion,

2) a primer A3 comprising at its 3'-end:

either a sequence homologous to a portion of the primer A2, not comprising the 3'-terminal nucleotide of A2,

or a sequence homologous to at least a portion of the first segment of primer A1,

wherein, when said primers A1 and A2 are both present, the 3'-end of the primer A1 hybridizes downstream of the 3'-end of the primer A2,

3) a primer B2 comprising at its 3'-end:

either a sequence homologous to all or part of the second segment of the primer B1, and comprising the 5' end of the sense sequence of said RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of said primer A1 or B1, but not comprising its 5' portion, and

4) a primer B3 comprising at its 3'-end:

either a sequence homologous to a portion of the primer B2, not comprising the 3'-terminal nucleotide of B2,

or a sequence homologous to at least a portion of the first segment of primer B1,

wherein, when said primers B1 and B2 are both present, the 3'-end of the primer B1 hybridizes downstream of the 3'-end of the primer B2.

7. Method of amplifying a sequence of a target nucleic acid, said sequence comprising, from its 5' end, in the 5'-3' direction, an upstream sequence having at least 5 nucleotides and from its 3' end in the 3'-5' direction, a downstream sequence having at least 5 nucleotides, and said target nucleic acid comprising the sequence to be amplified and extending, beyond the 3' end of said sequence to be amplified, via a downstream region, and beyond the 5' end of said sequence to be amplified, via an upstream region,

said method comprising bringing said target nucleic acid into contact with an excess amount of a set of primers, in the presence of a system having RNA polymerase activity, RNA-dependent DNA polymerase activity, DNA-dependent DNA polymerase activity and strand displacement activity, under conditions allowing the function of the activities, and in the presence of an excess amount of deoxyribonucleoside triphosphates and ribonucleoside triphosphates, such that said sequence of the target nucleic acid is amplified, said set of primers comprising:

a) at least one primer selected from the group consisting of:

1) a first primer comprising successively from its 5' end towards its 3' end:

a first optional polynucleotide segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of a first RNA polymerase promoter including its 3'-terminal portion,

and a third segment having the same length as said upstream sequence and being either homologous to said upstream sequence, or capable of hybridizing with said upstream sequence, and

2) a second primer comprising successively, from its 5' end towards its 3' end:

a first optional segment of an arbitrary sequence comprising at least 5 nucleotides,

a second segment comprising at least a portion of the sense sequence of a second RNA polymerase promoter including its 3'-terminal portion,

and a third segment having the same length as said downstream sequence and being either homologous to said downstream sequence, or capable of hybridizing with said downstream sequence,

wherein, when said first and second primers are both present, the third segment of one of the first and second primers is homologous to one of the upstream or downstream sequences of said sequence to be amplified and the third segment of the other primer is capable of hybridizing with the other downstream or upstream sequence,

b) at least one primer selected from the group consisting of:

1) a third primer comprising at its 3'-end:

either a sequence containing a segment that is homologous to all or part of the second segment of the first primer and containing the 5'-end of said sense sequence of said first RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of the first primer but not comprising its 5' end,

2) a fourth primer comprising at its 3'-end:

either a sequence containing a segment that is homologous to all or part of the second segment of the second primer and containing the 5'-end of said sense sequence of said second RNA polymerase promoter,

or a sequence homologous to a portion of the first segment of the second primer but not comprising its 5' end,

3) a fifth primer comprising at its 3'-end:

either a sequence homologous to a portion of the third primer, said portion not comprising the 3'-terminal nucleotide of said third primer,

or a sequence homologous to at least a portion of said first segment of the first primer,

wherein, when said third and fifth primers are both present, the 3'-end of the third primer hybridizes downstream of the 3'-end of the fifth primer, and

4) a sixth primer comprising at its 3'-end:

either a sequence homologous to a portion of the fourth primer, said portion not comprising the 3'-terminal nucleotide of the fourth primer,

or a sequence homologous to at least a portion of said first segment of the second primer,

wherein, when said fourth and sixth primers are both present, the 3'-end of the fourth primer hybridizes downstream of the 3'-end of the sixth primer, and

c) at least one primer selected from the group consisting of:

1) a seventh primer capable of hybridizing with said downstream region of the target nucleic acid, and

2) an eighth primer capable of hybridizing with a sequence complementary to said upstream region of the target nucleic acid.

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L11: Entry 12 of 25 File: USPT Feb 6, 2001

DOCUMENT-IDENTIFIER: US 6183998 B1
TITLE: Method for reversible modification of thermostable enzymes

Brief Summary Text (7):
The principle of PCR is basically described by the amplification of specific nucleic acid sequences of a nucleic acid target template, using at least one, two or several target-specific oligonucleotides (primers), a thermostable nucleic acid polymerase, deoxyribonucleoside triphosphates and a reaction buffer. DNA synthesis initiates at the accessible 3 '--OH group of the target-specific oligonucleotides flanking the DNA sequence to be copied, thereby generating an identical copy of the target template nucleic acid sequence. The enzymatic reaction is repeated for a substantial number of thermal cycles consisting of the denaturation of the target nucleic acid, annealing of the primer oligonucleotides to complementary nucleic acid sequences and the subsequent extension of these primer-template complexes using a thermostable nucleic acid polymerase in a sequence-dependent manner. Hybridization of primers occurs usually at sufficiently high temperature to provide annealing conditions that ensure binding of the oligonucleotide primers mainly to its complementary target nucleic acid sequence. However, PCR reaction mixtures are often assembled at room temperature, thus providing much less stringent oligonucleotide hybridization conditions, at a temperature at which most thermostable nucleic acid polymerases possess DNA synthesis activity. Since non-specifically annealed and extended oligonucleotides lead to formation of non-specific amplification products, these mis-primed non-specific PCR products can compete during subsequent PCR cycles with the specific PCR product for primer molecules, polymerase and nucleotides, thereby severely interfering or even abrogating the amplification reaction of the specific amplification product (see, Chou et al., Nucleic Acids Research, 20(7): 1717-1723 (1992)).

Brief Summary Text (10):
Extension of mis-primed oligonucleotides can also be prevented by pre-incubating the primers with a compound that binds specifically to single-stranded DNA in a heat-reversible manner, such as a single-strand binding protein. Such a compound would prevent the oligonucleotide primer from hybridizing to any template sequence at ambient temperature. For instance, the use of Gene 32 protein, a single stranded DNA binding protein, was shown to improve the yield of PCR products in Schwarz et al., Nucleic Acid Research, 18(4): 10 (1990).

Brief Summary Text (20):
and other zwitterionic bases characterized by the .sup.-- OOC--CH.sub.2 --NMe.sub.3.sup.+ group (collectively "betaines"). Additional PCR additives include multifunctional polyols, preferably trifunctional polyols, most preferably glycerol; amides, preferably carbamides, most preferably formamide; alkaline ammonia salts, preferably alkylated ammonia salts, most preferably tetramethylammonium chloride; sulfoxides, preferably alkylated sulfoxides, most preferably dimethylsulfoxide; sulfates, preferably inorganic sulfates, most preferably ammonium sulfate polyalkylene glycols, most preferably polyethylene glycol. Additionally, SSB protein (single strand binding protein), preferably E. coli SSB protein, T4 gene 32 protein, yeast SSB protein, may also be used. Preferred PCR additives also include calf thymus protein UP1.

Brief Summary Text (21):
In another aspect, the present invention relates to the use of RNase H positive and RNase H negative reverse transcriptases in combination with inactivated Taq DNA polymerase for a continuous reverse transcription polymerase chain reaction (RT-PCR) to be performed in a single reaction tube without interrupting the enzymatic reactions by additional handling steps.

Brief Summary Text (29):
Aldehydes useful as modifying reagents for reversibly inactivating thermostable enzymes according to this invention will have the general formula RHC.dbd.O, where R is H or an alkyl, aryl, or alkylaldehyde (O.dbd.CH--(CH.sub.2).sub.0-9 --) group of 1 to 10 carbon atoms. Such reagents include formaldehyde, acetaldehyde, glutaraldehyde, and the like, however, because the aldehyde is believed to inactivate the enzyme by forming crosslinks between enzyme molecules, formaldehyde is especially preferred. The aldehyde employed must be at least partially soluble in water and capable of reacting below about 50.degree. C. with the thermostable enzyme to form a modified enzyme that will return at least part of its former enzymatic primer extension activity after incubation at a high temperature, i.e., above about 50.degree. C. It is believed that reaction of the enzyme with aldehyde reagent forms heat-labile crosslinks through amino-functional side-chains, and therefore any aldehyde compound that is reactive with an enzyme to be treated and which forms such crosslinks without significantly degrading the enzyme or irreversibly inactivating the enzyme is suitable. The preferred reagent for the reversible inactivation of thermostable DNA polymerases for the use in primer extension reactions is formaldehyde.

Brief Summary Text (33):
Crosslinking of the enzyme can have additional stabilizing effects. Various physical studies have shown that the action of formaldehyde on proteins under conditions close to physiological conditions may produce intramolecular crosslinks hindering denaturation of proteins. Reductive alkylation with formaldehyde has little or no effect on the distribution of charged groups and causes a minimal disturbance of electrostatic interactions (see, Means, Methods in Enzymology, 47: 469-78 (1977)). Formaldehyde treatment, e.g., to fix or inactivate enzyme activity, has been used for a number of purposes. Formaldehyde has been used more extensively than other aldehydes in industry, research and medicine, e.g., for preparation of vaccines, inactivation of viral RNA, for making direct functional changes of nucleic acids in vivo, and for the investigation through modification of structural and functional characteristics of DNA and proteins (Feldman, Progr. Nucl. Acid Res. & Mol. Biol., 13: 1-49 (1973)).

Brief Summary Text (34):
As described in EP 0 771 870 A1, reaction of most acylating agents, e.g., citraconic anhydride, with protein amino-groups is competitive with hydrolysis of the reagent by water or hydroxyl ions. The reaction conditions must be adjusted to take this into account, and non-aqueous solutions may be required for optimal performance. The optimal conditions employ a high concentration of the anhydride reagent at a pH that maximizes deprotonation of amino-groups but minimizes hydrolysis. The rapid liberation of acid during the reaction must also be taken into account, especially during the long incubation times, which are typical for the reaction (Atassi et al., Methods in Enzymology, 25(B): 546-53 (1972); Butler et al., Methods in Enzymology. 25(B): 191-199 (1972)).

Brief Summary Text (36):
As mentioned above, the primary sites of formaldehyde treatment appear to be the .epsilon.--NH.sub.2 group of lysine, e.g., in the histones. This group has a pK of 11.3 and at pH 7 is fully protonated and highly reactive. The rate of formaldehyde crosslinking of histones with DNA is very rapid (80% crosslinked within 1 min.). The dramatic decrease of ribonuclease activity is achieved after a few minutes upon addition of formaldehyde (Jackson, Cell, 15(3): 945-54 (1978)). The reaction of formaldehyde with an amine is thought to be an essential step. At the same time there is no consensus on the exact mechanism of formaldehyde action on the amino-groups of a protein. No evidence for so-called Schiff base (R--N.dbd.CHR) formation was obtained in the reaction of formaldehyde with .alpha.-amino groups of amino acids although reductive alkylation of amino groups in protein by formaldehyde in the presence of pyridine borane and borohydride propose the mechanism of Schiff-formation.

Brief Summary Text (38):
In a preferred method, DNA polymerase purified from Thermus aquaticus is incubated with up to 2 M formaldehyde, preferably with 20 mM to 1 M formaldehyde, at 37.degree. C. in a water bath, for about 1 minute to 60 minutes, preferably 30 minutes. Subsequently, the reaction mixture is chilled on ice and the residual formaldehyde is removed from the formaldehyde-treated DNA polymerase by ultra-filtration and by washing in a buffer consisting of 20 mM Hepes pH 7.9, 1 mM EDTA, 200 mM KCl. The

treated DNA polymerase is then dialyzed overnight at 4.degree. C. against a storage buffer consisting of 20 mM Tris pH 9.0, 0.1 mM EDTA, 0.5% (v/v) Tween 20, 10 mM ethanolamine, 100 mM KCl, 1 mM DTT, 0.5% (v/v) Nonidet P-40, 50% (v/v) Glycerin. Surprisingly, it was found that formaldehyde treatment of thermostable nucleic acid polymerase isolated from either Thermus aquaticus or Pyrococcus furiosus, cloned and overexpressed in Escherichia coli, under essentially physiological conditions gives a stable product after removal of unreacted formaldehyde.

Brief Summary Text (40):
In addition to using the modified, inactivated nucleic acid polymerases as described above, PCR reactions may be improved by using additives that affect the melting behavior of nucleic acids in the reaction mixture. For example, difficult PCR amplifications, such as reactions that yield non-specific products, and especially amplification of templates having a high GC content or having extensive secondary structure, may be improved by employing additives that "isostabilize" AT- and GC-base pairing to the level of AT-base pair stability. Suitable such PCR additives include multifunctional polyols, preferably trifunctional polyols, most preferably glycerol; amides, preferably carbamides, most preferably formamide; alkaline ammonia salts, preferably alkylated ammonia salts, most preferably tetramethylammonium chloride; sulfoxides, preferably alkylated sulfoxides, most preferably dimethylsulfoxide; sulfates, preferably inorganic sulfates, most preferably ammonium sulfate polyalkylene glycols, most preferably polyethylene glycol. Additionally, SSB protein (single strand binding protein), preferably E. coli SSB protein (see, Schwarz et al., E. coli SSB protein, Nucleic Acids Research, 18: 1079 (1990)), T4 gene 32 protein, or yeast SSB protein, may also be used. Preferred PCR additives also include calf thymus protein UP1 (see, Amrute et al., Biochemistry, 33(27): 8282-8291 (1994).

Brief Summary Text (41):
A particularly preferred PCR additive for this purpose is betaine (1-carboxy-N,N,N-trimethyl-methanaminium inner salt) and other zwitterionic bases characterized by the .sup.-- OOC--CH.sub.2 --NMe.sub.3.sup.+ group (collectively "betaines").

Brief Summary Text (42):
The PCR additives are advantageously added to a PCR reaction mixture in an amount effective to improve the specificity of the amplified product. Typically concentrations of additive from 1 mM to 5M, preferably about 1M, are used, however any amount that improves the yield of the specific amplification product, compared with a PCR reaction carried out in the absence of the additive, is suitable.

Brief Summary Text (43):
In PCR reactions involving reverse transcription (RT-PCR), the materials and methods of the present invention permit a continuous reaction to be carried out in one vessel, without interrupting the enzymatic reactions by additional handling steps.

Detailed Description Text (15):
This example describes the modification of Taq DNA polymerase with formaldehyde. Taq DNA polymerase was modified in a buffer containing 200 mM KCl; 1 mM EDTA; 20 mM HEPES; pH 7.9 at enzyme concentrations of 10-20 U/.mu.l. Formaldehyde was purchased from Merck as a 37% stock solution. Immediately before use, 100 .mu.l of formaldehyde were diluted with 168 .mu.l of distilled water to yield a formaldehyde stock solution. Different amounts (40 .mu.l to 800 .mu.l) of this stock solution were mixed with 4 ml of Taq DNA polymerase to achieve the following end concentrations of formaldehyde: 0.2.times. stock solution (referred to as 0.2.times.); 0.18.times. stock solution; 0.16 x stock solution; 0.14.times. stock solution; 0.12.times. stock solution; 0.1.times. stock solution; 0.05 x stock solution; 0.03 x stock solution; 0.02 x stock solution; 0.01 x stock solution. The mixture was incubated for 30 min. at 37.degree. C. in a temperature-controlled water bath, followed by quick chilling of the sample on ice and removing residual formaldehyde either by gel filtration or by ultrafiltration. To stabilize the formed complex, the modified enzyme was dialysed against a storage buffer containing 100 mM KCl; 0.1 mM EDTA; 0.5% (v/v) Tween 20; 0.5% (v/v) Nonidet P-40; 1 mM DTT; 10 mM ethanolamine; 50% glycerol; 20mM Tris/Cl; pH 9.0.

Detailed Description Text (18):
This example shows that primer extension activity of Taq DNA polymerase can be reduced

by formaldehyde treatment and subsequently recovered by incubation at high temperature. The reduction of enzyme activity depends on the concentration of the formaldehyde and is not caused by the presence of formaldehyde itself in the primer extension reaction. The primer extension activity of the treated enzyme was recovered by heating at 90.degree. C. for 30 min. The procedure describes an easy way to optimize the concentration and duration of formaldehyde treatment.

Detailed Description Text (19):
1 .mu.l of Taq DNA polymerase (100 units) in above described storage buffer without detergents was treated with 10 .mu.l in a final concentration of 0.3, 1 and 3 M of formaldehyde (Aldrich) for 10 min. at 37.degree. C. then chilled on ice for 10 min. and diluted with 189 .mu.l TE buffer (10 mM Tris HCl, pH 9.0 at 25.degree. C., 1 mM EDTA). As a control, 1 .mu.l of non-treated enzyme was mixed with 10 .mu.l of formaldehyde solution to the final concentration of 0.3, 1 and 3 M of formaldehyde and immediately diluted (without heating) with 189 .mu.l TE buffer to the required concentration.

Detailed Description Text (23):
Effect of Formaldehyde Concentration Used to Inactivate Taq DNA Polymerase on Primer Extension Activity

Detailed Description Text (24):
This example describes the effect of formaldehyde concentration employed to inactivate Taq DNA polymerase on recovery of enzyme activity, as measured in a primer extension reaction as described in Example 1. Reaction mixtures containing either 0.01.times., 0.02.times., 0.03.times., 0.05.times., 0.1.times., 0.18.times. Taq DNA polymerase were preincubated for 15 minutes at 95.degree. C. Negative control contained no DNA polymerase. Assays were performed as described in Example 1, and the results below show relative primer extension activities of indicated Taq DNA polymerases normalized to 0.01.times. Taq DNA polymerase. Values indicate average activities determined from duplicate samples.

Detailed Description Text (25):
The results demonstrate that recovery of enzyme activity is dependent on the formaldehyde concentration used to inactivate Taq DNA polymerase. However, 0.03.times. Taq DNA polymerase and Taq DNA polymerase preparations treated with higher formaldehyde concentrations showed no significant further decrease of primer extension activity.

Detailed Description Text (56):
Pfu DNA polymerase was modified in exactly the same way as described for Taq DNA polymerase in Example 3, with the exception that the enzyme was used at concentrations of 2-5 U/.mu.l.

Detailed Description Text (74):
Continuous Reverse Transcription Polymerase Chain Reaction (RT-PCR) using a Combination of Reverse Transcriptase and Inactivated Taq DNA Polymerase

Detailed Description Text (75):
This example describes the use of inactivated Taq DNA polymerase in combination with a reverse transcriptase for a continuous reverse transcription polymerase chain reaction (RT-PCR) in a single reaction tube without interrupting the enzymatic reactions by additional handling steps.

Detailed Description Text (77):
Coupled reverse transcriptase and PCR reactions were performed in single tubes using 0.03x Taq DNA polymerase and either avian myeloblastosis virus reverse transcriptase (AMV, Boehringer Mannheim) or a RNase H negative form of the Moloney murine leukaemia virus reverse transcriptase (SUPERSCRIPT.TM. II, Life Technologies) 0.5 .mu.l of 0.03.times. Taq DNA polymerase was used in each reaction as well as either 5 units AMV or 100 units SUPERSCRIPT.TM. II. A fragment of the human glycerinaldehyde-3-phosphate-dehydrogenase gene was reverse transcribed from total RNA into complementary DNA (cDNA) by either reverse transcriptase and subsequently amplified by 0.03.times. Taq DNA polymerase using primers flanking a 831 bp PCR fragment including primer sequences (24-mer 5'-ATGGGGAAGGTGAAGGTCGGAGTC-3' (SEQ ID NO:

8), 24-mer 5'-AGTGTAGCCCAGGATGCCCTTGAG-3' (SEQ ID NO: 9)) for both enzymatic reactions. The reaction mixture contained 1 .mu.g total RNA from HeLa cells (purified from human HeLa cell line using RNeasy.RTM. Maxi Kit, QIAGEN, Hilden, Germany), 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl.sub.2, 200 .mu.M of each dNTP, 0.4 .mu.M of each primer. Final reaction volume was 50 .mu.l. The thermal cycling profile consisted of a 30 min. lasting reverse transcriptase step at 50.degree. C., directly followed by a pre-incubation step at 95.degree. C. for 15 min. during which simultaneously the respective reverse transcriptase became inactivated. This step was followed by 40 cycles: denaturation step for 30 sec. at 94.degree. C., annealing step for 45 sec. at 55.degree. C. and an extension step for 1 min. at 72.degree. C. Amplification products were analyzed on a 1% agarose gel by gel electrophoresis using a 1.times. TAE electrophoresis buffer and a DNA intercalating dye. Gel electrophoresis was performed for approximately 35 min. at 85 volts. Ethidium bromide-stained amplification products were visualized using UV irradiation.

Detailed Description Text (78):
The results demonstrate that formaldehyde-inactivated Taq DNA polymerase can be used in combination with reverse transcriptases in a continuous single tube RT-PCR reaction. Advantageously, formaldehyde-inactivated Taq DNA polymerase is inactive during the reverse transcriptase reaction and only becomes active for the PCR reaction, while during the heat activation step, residual reverse transcriptase activity is destroyed. This advantageous effect of formaldehyde-inactivated Taq DNA polymerase can be used in continuous single tube RT-PCR with both RNase H positive and RNase H negative reverse transcriptases.

Other Reference Publication (19):
Shaffer A.L. et al, Amplification, detection, and automated sequencing of Gibbon Interleukin-2 mRNA by Thermus aquaticus DNA polymerase reverse transcription and polymerase chain reaction, Analytical Biochem., vol. 190, Nov. 1, 1990, pp 292-296.

CLAIMS:

1. A modified thermostable DNA polymerase, produced by a reaction, under essentially aqueous conditions, of a thermostable DNA polymerase and a modifier reagent at a temperature of less than 50.degree. C., wherein said modifier reagent is an aldehyde of the formula RHC.dbd.O, where R is H or an alkyl, aryl, or alkylaldehyde group of 1 to 10 carbon atoms, and wherein said reaction results in a thermally reversible inactivation of the DNA polymerase.

7. The modified thermostable DNA polymerase according to claim 1, wherein said polymerase is derived from an organism selected from the group consisting of Thermus aquaticus, Thermus thermophilus, Thermus flavus, Pyrococcus furiosus, Pyrococcus woesei, Pyrococcus spec. (strain KOD1), Pyrococcus spec. GB-D, Thermococcus litoralis, Thermococcus sp. 9.degree. N-7, Thermotoga maritima, Pyrococcus spec. ES4 (endeavori), Pyrococcus spec. OT3 (horikoshii),Pyrococcus profundus, Thermococcus stetteri, thermococcus spec. AN1 (zilligii), Thermococcus peptonophilus, Thermococus celer, and Thermococcus fumicolans.

8. The modified thermostable DNA polymerase according to claim 1, wherein said polymerase is derived from an organism selected from the group consisting of Thermus aquaticus, Pyrococcus furiosus, and Pyrococcus woesei.

11. A mixture according to claim 10, wherein said PCR additive is selected from the group consisting of betaines, multifunctional polyols, amides, alkaline ammonia salts, sulfoxides, sulfates, SSB proteins, and calf thymus protein UP1.

12. A mixture according to claim 11, wherein said PCR additive is selected from the group consisting of betaines, glycerol, formamide, tetramethylammonium chloride, dimethylsulfoxide, polyethylene glycol, E. coli SSB protein, T4 gene 32 protein, yeast SSB protein, and calf thymus protein UP1.

16. A reagent kit according to claim 15, wherein said PCR additive is selected from the group consisting of betaines, multifunctional polyols, amides, alkaline ammonia salts, sulfoxides, sulfates, SSB proteins, and calf thymus protein UP1.

17. A reagent kit according to claim 16, wherein said PCR additive is selected from the group consisting of betaines, glycerol, formamide, tetramethylammonium chloride, dimethylsulfoxide, polyethylene glycol, E. coli SSB protein, T4 gene 32 protein, yeast SSB protein, and calf thymus protein UP1.

21. A reagent mixture according to claim 20, wherein said PCR additive is selected from the group consisting of betaines, multifunctional polyols, amides, alkaline ammonia salts, sulfoxides, sulfates, SSB proteins, and calf thymus protein UP1.

22. A reagent mixture according to claim 21, wherein said PCR additive is selected from the group consisting of betaines, glycerol, formamide, tetramethylammonium chloride, dimethylsulfoxide, polyethylene glycol, E. coli SSB protein, T4 gene 32 protein, yeast SSB protein, and calf thymus protein UP1.

24. A method for the amplification of a target nucleic acid comprising the step of:

(a) contacting said nucleic acid with an amplification reaction mixture containing a modified thermostable enzyme, wherein said modified thermostable enzyme is produced by a reaction, under essentially aqueous conditions, of a thermostable enzyme and a modifier reagent at a temperature of less than 50.degree. C., wherein said modifier reagent is an aldehyde of the formula RHC.dbd.O, where R is H or an alkyl, aryl, or alkylaldehyde group of 1 to 10 carbon atoms, and wherein said reaction results in thermally reversible inactivation of the enzyme.

29. The method of claim 27, wherein said polymerase is derived from an organism selected from the group consisting of Thermus aquaticus, Thermus'thermophilus, Thermus flavus, Pyrococcus furiosus, Pyrococcus woesei, Pyrococcus spec. (strain KOD1), Pyrococcus spec. GB-D, Thermococcus litoralis, Thermococcus sp. 9.degree. N-7, Thermotoga maritima, Pyrococcus spec. ES4 (endeavori), Pyrococcus spec. OT3 (horikoshii), Pyrococcus profundus, Thermococcus stetteri, Thermococcus spec. AN1 (zilligii), Thermococcus peptonophilus, Thermococcus celer, and Thermococcus fumicolans.

33. The method of claim 32, wherein said PCR additive is selected from the group consisting of betaines, multifunctional polyols, amides, alkaline ammonia salts, sulfoxides, sulfates, SSB proteins, and calf thymus protein UP1.

34. The method of claim 33, wherein said PCR additive is selected from the group consisting of betaines, glycerol, formamide, tetramethylammonium chloride, dimethylsulfoxide, polyethylene glycol, E. coli SSB protein, T4 gene 32 protein, yeast SSB protein, and calf thymus protein UP1.

36. A polymerase chain reaction amplification mixture comprising:

(a) a modified thermostable enzyme according to claim 1;

(b) a set of polymerase chain reaction specific primers;

(c) a reverse transcriptase; and

(d) a nucleic acid.

38. The reaction mixture of claim 36 wherein said reverse transcriptase is RNase H positive or RNase H negative.

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